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14. ABSTRACT

The most significant finding during the research period (max 200 words)
purpose, scope, major findings and be an **up-to-date** report of the progress in terms of results and significance. Subject terms are keywords that may have been previously assigned to the proposal abstract or are keywords that may be significant to the research

The goal of this project was to determine the extent to which six drugs reduce or normalize the pathologically high mTORC1 activity seen in TSC-defective cells, to determine whether they can improve other abnormal phenotypes described for TSC-defective cells, and to select a candidate for proof of concept in animal studies. The effect of the drugs on mTORC1 signaling, cell viability, serum-independent proliferation, p27 nuclear localization, cell motility, cell morphology, cell size, cell-cell contacts and focal adhesions were determined in $TSC2^{+/+}$ and $TSC2^{-/-}$ mouse embryo fibroblasts. Two drugs, nitazoxanide and tizoxanide, did not significantly inhibit mTORC1 in these cells. Four additional drugs, perhexiline, amiodarone, dronedarone and niclosamide were able to reverse the abnormal epithelial-like morphology of TSC2-defective fibroblasts to a more normal fibroblastic morphology, which may indicate therapeutic potential.

15. SUBJECT TERMS
mTOR, cytotoxicity, TSC, cell size, cell morphology, perhexiline, amiodarone, dronedarone, niclosamide

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Introduction

Tuberous sclerosis is a disease associated with abnormally high mTORC1 signaling. The goal of this research was to determine the extent to which five approved drugs and one active drug metabolite that are mTOR signaling inhibitors (niclosamide, perhexiline, amiodarone, dronedarone, nitazoxanide and its active metabolite tizoxanide,) can reduce or normalize the high mTORC1 activity seen in TSC-defective cells and can ameliorate other abnormal phenotypes found in TSC-defective cells, and to select the best drug candidate to determine whether mTORC1 inhibition and inhibition of the growth of TSC-defective cells can be achieved in vivo at tolerated drug doses.

Body

Specific Aim 1.1. Normalization of mTORC1 signaling in TSC-defective cells.

In previous experiments (Balgi, 2009), we had tested the effect of short-term exposure (4 h) to niclosamide, perhexiline, and amiodarone on mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The amiodarone analog dronedarone was only approved by the FDA in 2009. We have now compared the effect of amiodarone and dronedarone on mTORC1 activity in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. Dronedarone effectively reduced mTORC1 signaling at 3 μM and higher concentrations as shown by reduction of the phosphorylation of the mTORC1 substrate S6 kinase at threonine 389, and by its increased electrophoretic mobility (Figure 1). It is considerably more potent than amiodarone, which inhibits mTORC1 signaling in the 10-30 μM concentration range (Figure 1). This observation is relevant because dronedarone appears to be a substantially safer drug than amiodarone in humans.

Nitazoxanide and tizoxanide inhibit mTORC1 signaling in MCF-7 human breast cancer cells and THP1 human leukemia cells (Lam, 2012). We have tested them on $TSC2^{+/+}$ and $TSC2^{-/-}$ mouse embryo fibroblasts. Neither drug showed significant inhibition of mTORC1 signaling in these cells (Figure 2). Nitazoxanide and tizoxanide were therefore excluded as potential therapeutics for tuberous sclerosis and they were not studied further.

We next tested the effect of niclosamide, perhexiline, amiodarone and dronedarone on mTORC1 signaling during longer-term exposure (72h). By 72 h, mTORC1 signaling had returned to levels seen in untreated cells for all drugs. By contrast, the irreversible mTORC1 inhibitor rapamycin used here as a positive control showed complete mTORC1 inhibition after 72 h exposure (Figure 3). The results indicate that mTORC1 inhibition by these four drugs is reversible and that the drugs may be metabolically inactivated during long term exposure, or that the cells employ compensatory mechanisms to restore mTORC1 activity during long term drug treatment.

We next tested the effect of the drugs during much longer-term exposure (3 weeks). Lysates were analysed at weekly intervals during drug exposure. The drugs induced a variable degree of decrease in mTORC1 signalling in $TSC2^{-/-}$ cells after 1 week exposure. However, after 3 weeks no significant inhibition was observed in $TSC2^{-/-}$ cells, indicating that the cells were adapting to treatment. mTORC1 signalling remained profoundly inhibited during 3-week treatment with rapamycin (Figure 4).

We also determined the effects of the drugs on cell viability. There was no significant reduction in cell viability during 4 h exposure to the drugs except at high concentrations of perhexiline and dronedarone (10 μM; Figure 5).

Therefore, during 4 h exposure, niclosamide, amiodarone and dronedarone significantly inhibited mTORC1 signaling in $TSC2^{-/-}$ cells at concentrations that are not toxic (10 μM niclosamide, 30 μM amiodarone and 3 μM dronedarone). By contrast, perhexiline was toxic to cells at the concentration that inhibits mTORC1 signaling (10 μM), indicating that it is less promising as a drug candidate for treatment of tuberous sclerosis.

The effect of the drugs on cell viability was also measured during longer term exposure of 5 days. Both $TSC2^{+/+}$ and $TSC2^{-/-}$ cells showed reduced viability compared with untreated controls following treatment with niclosamide above 0.3 μM, perhexiline above 3 μM, amiodarone above 1 μM and dronedarone above 1 μM (Figures 6 and 7). Combined, these results indicate that the drugs do not kill cells but reduce their proliferation, which is expected of mTORC1 inhibitory drugs.

Specific Aim 1.2. Amelioration of abnormal phenotypes associated with the TSC defect.

A number of morphological and biochemical abnormalities have been reported in cultured TSC-defective cells. We examined whether the four drugs could ameliorate any of these abnormalities.

1- Serum-independent proliferation
It has been reported that $TSC2^{-/-}$ cells are able to proliferate in medium lacking serum while $TSC2^{+/+}$ cells are unable to do so. We observe that $TSC2^{+/+}$ cells are more dependent on serum for proliferation than $TSC2^{-/-}$ cells (Figure 8). However, the proliferation of $TSC2^{-/-}$ cells was not completely independent of serum, rather the cells proliferated at a lower rate in the absence of serum than in its presence (Figure 8).

To determine whether the drugs reduced this difference and made $TSC2^{-/-}$ cell proliferation more sensitive to serum withdrawal, we treated $TSC2^{+/+}$ cells and $TSC2^{-/-}$ cells with the drugs in the absence or presence of serum and viable cells were measured at 48 h (Figures 9 and 10). The decreased sensitivity of $TSC2^{-/-}$ cells to serum withdrawal was not corrected by exposure to niclosamide (Figure 9) at low concentrations that did not reduce the viability or proliferation of both cell lines. Higher concentrations of niclosamide caused reduced viability in both cell lines in serum and without serum. The other drugs also did not correct this defect (Figure 10).

2-Reduced p27 expression and nuclear localization
It has been reported that TSC2-defective fibroblasts have reduced levels of the protein p27 and mislocalization of p27 from the nucleus to the cytoplasm. We used quantitative automated immunofluorescence microscopy to examine whether the drugs cause increased localization of p27 to the nucleus. Cells were treated with the drugs for 4 h, after which they

were fixed with paraformaldehyde and incubated with p27 antibody followed by a fluorescently labeled secondary antibody. In the absence of drug treatment, p27 was localized to the cytoplasm and the nucleus in both cell lines (Figure 11).

To provide a quantitative measure, nuclear fluorescence intensity was determined in many cells in duplicate wells. Table 1 shows the average p27 fluorescence intensity (arbitrary fluorescence units) and the number of nuclei measured for each well and condition. Niclosamide caused an increase in nuclear p27 but this increase was more pronounced in $TSC2^{+/+}$ cells than in $TSC2^{-/-}$ cells. Dronedarone caused an increase in nuclear p27 in both $TSC2^{-/-}$ and $TSC2^{+/+}$ cells, and only at high concentrations that reduce cell viability. Amiodarone has not been tested.

3- Reduced cell motility

TSC2-defective cells are reported to have reduced motility. We have used two assays to measure the effect of drugs on cell motility: a wound healing assay and a fluorescent bead track clearing assay. Both assays confirmed previous reports that TSC2-defective cells have reduced motility. In the wound healing assay, cells were cleared from a confluent monolayer and the width of the cleared zone (the wound) was measured over time. 5.5 h after wound generation $TSC2^{+/+}$ cells had migrated into the gap left by the wound more than $TSC2^{-/-}$ cells (Figures 12, 13, 14). In the fluorescent bead track clearing assay, a uniform lawn of small fluorescent beads is deposited onto a cell culture plastic surface. Cells are then added and as they move along the surface, they phagocytose the beads, leaving a non-fluorescent track whose area can be measured by automated fluorescence microscopy. The tracks of $TSC2^{-/-}$ cells were smaller than those of $TSC2^{+/+}$ cells (Figures 15, 16 and 17).

In the wound healing assay, drugs were added and the width of the cleared zone (the wound) was measured over time. In both $TSC2^{+/+}$ and $TSC2^{-/-}$ cells, perhexiline, niclosamide and dronedarone did not affect wound healing but amiodarone significantly inhibited wound healing (Figures 13 and 14)

In the bead clearing assay, the drugs had little or no effect on cell motility of either cell line except for nitazoxanide (10 μM) which increased the motility of $TSC2^{-/-}$ cells (Figures 15, 16 and 17).

4- Membrane ruffling defects

Membrane ruffling is a process that occurs at the leading edge of cells while they migrate. $TSC2^{-/-}$ cells have been reported to display reduced ruffling activity. Membrane ruffling is observed under the light microscope and is not a quantitative assay. Its main purpose here was to confirm results obtained in the cell motility assays. However, none of the drugs increased the migration of $TSC2^{-/-}$ cells in both the wound healing and the bead clearing assays. For this reason, the effects of the compounds on membrane ruffling were not tested.

5- Reduced PDGF-PI3K-Akt signaling

This task has not yet been carried out.

6. Cell morphology

The immortalized $TSC2^{+/+}$ mouse embryo fibroblasts display a typical fibroblastic morphology: they are bipolar or multipolar, have an elongated shape, tend to grow as individual cells and do not make extensive contact with neighboring cells in non-confluent cultures. By contrast the immortalized $TSC2^{-/-}$ mouse embryo fibroblasts have an abnormal morphology that is more epithelial-like. They are very large and flat and round or polygonal in shape, with more regular dimensions than fibroblasts and they grow in patches with extensive cell-cell contacts. Unlike $TSC2^{+/+}$ cells, individual $TSC2^{-/-}$ cells are rarely seen in non-confluent cultures (Figure 18).

$TSC2^{+/+}$ and $TSC2^{-/-}$ cells were treated with rapamycin, dronedarone, amiodarone or niclosamide for up to 7 days to determine whether the drugs elicited a change in morphology. The drugs did not cause any significant change in $TSC2^{+/+}$ cell morphology (Figure 19). Interestingly, they caused a very clear morphological change in $TSC2^{-/-}$ cells, which became much more fibroblastic-like, resembling the morphology of $TSC2^{+/+}$ cells (Figure 19). Rapamycin, amiodarone and niclosamide caused cells to adopt a highly fibroblastic morphology while dronedarone caused a partial reversion of the phenotype, with cells having a hybrid epithelial-fibroblastic appearance. Perhexiline at 3μM,did not produce any visible change in morphology, although increasing the concentration to 5 μM did reverse the phenotype (Figure 20).

Visual examination of Figure 19 shows that although the drug-treated $TSC2^{-/-}$ cells took on a fibroblastic appearance, they clearly remained larger than $TSC2^{+/+}$ cells. To examine cell size quantitatively, cells were treated with the drugs for 7 days, and then stained with rhodamine-phalloidin to visualize F-actin, which fills the cytoplasm and can be used to determine total cell area. A Cellomics Arrayscan VTI fluorescence imager was used to quantify the average area of the cells. Untreated $TSC2^{-/-}$ cells are roughly three-times larger than untreated $TSC2^{+/+}$ cells (Figure 21). Rapamycin, niclosamide and amiodarone caused a reduction in the size of the $TSC2^{-/-}$ cells (Figure 20) while dronedarone and perhexiline had little effect. However, the cells remained larger than $TSC2^{+/+}$ cells. Interestingly, the drugs did not cause a change in the size of $TSC2^{+/+}$ cells (Figure 21).

7. Cell-cell contacts

We have observed that $TSC2^{-/-}$ cells grow in patches with apparent extensive cell-cell contacts, while $TSC2^{+/+}$ cells are much more spread out and show less extensive cell-cell contacts. Upon 7 day treatment with drugs, $TSC2^{-/-}$ cells also showed fewer cell-cell contacts than untreated $TSC2^{-/-}$ cells at a similar confluency (Figure 19). $TSC2^{+/+}$ cells did not display any change in cell-cell contacts upon treatment with drugs (Figure 19).

To visualize changes in cell-cell contacts, cells were treated with drugs for 6 days and immunofluorescence microscopy was performed using an antibody to the cell-cell adhesion protein β-catenin, and using DRAQ5 as a nuclear stain. At similar confluencies, untreated $TSC2^{-/-}$ cells showed more cell-cell contact than untreated $TSC2^{+/+}$ cells, as seen by the bright and extensive β-catenin localization at cell-cell junctions (Figure 21, top right). Upon treatment with drugs, the $TSC2^{-/-}$ cells showed a more fibroblastic appearance and a

marked decrease in cell-cell contacts compared to untreated cells at a similar confluency (Figure 22). Even after exposure of $TSC2^{-/-}$ cells to 2μM dronedarone, which did not cause a large change in morphology, fewer cell-cell attachments were observed and cells were more dispersed (Figure new 22?). Therefore, long-term treatment with drugs disrupted the extensive cell-cell contacts seen in $TSC2^{-/-}$ cells . The drugs did not seem to affect the cell-cell contacts between $TSC2^{+/+}$ cells (Figure 22).

8. Focal adhesions

It has been documented that the TSC2 gene product tuberin plays a role in regulation of focal adhesions (Astrinidis, 2002). We are currently in the process of optimizing focal adhesion detection using vinculin immunofluorescence microscopy. Preliminary results show that $TSC2^{-/-}$ cells display more focal adhesions than $TSC2^{+/+}$ cells (Figure 23). Experiments are being performed to determine whether long-term treatment with the drugs will alter the amount of focal adhesions in either $TSC2^{+/+}$ or $TSC2^{-/-}$ cells.

9. Primary cilium.

The single, non-motile cilium found on a variety of vertebrate cells known as the primary cilium has recently been found to be implicated in a variety of diseases such as polycystic kidney disease, where a defect in the functioning of primary cilia has been shown. It has also been reported that TSC1 and TSC2 regulate the formation of the primary cilium, and that $TSC2^{-/-}$ mouse embryo fibroblasts are more likely to contain a primary cilium than $TSC2^{+/+}$ mouse embryo fibroblasts, and with increased length (Hartman, 2009). We induced primary cilium formation by serum-deprivation for 24 h, after which cells were incubated on ice to depolymerize microtubules, and then incubated with an antibody against acetylated tubulin to detect the primary cilium. This protocol is currently being optimized, but to date we have been able to visualize the primary cilium in both $TSC2^{+/+}$ and $TSC2^{-/-}$ cells (Figure 24). Experiments are being performed to determine whether long-term treatment with the drugs will alter the frequency or length of primary cilium in either $TSC2^{+/+}$ or $TSC2^{-/-}$ cells.

List of personnel receiving pay:
Anderson, Hilary
Balgi, Aruna
Mendes, John
Moosa, Alym
Zimmerman, Carla

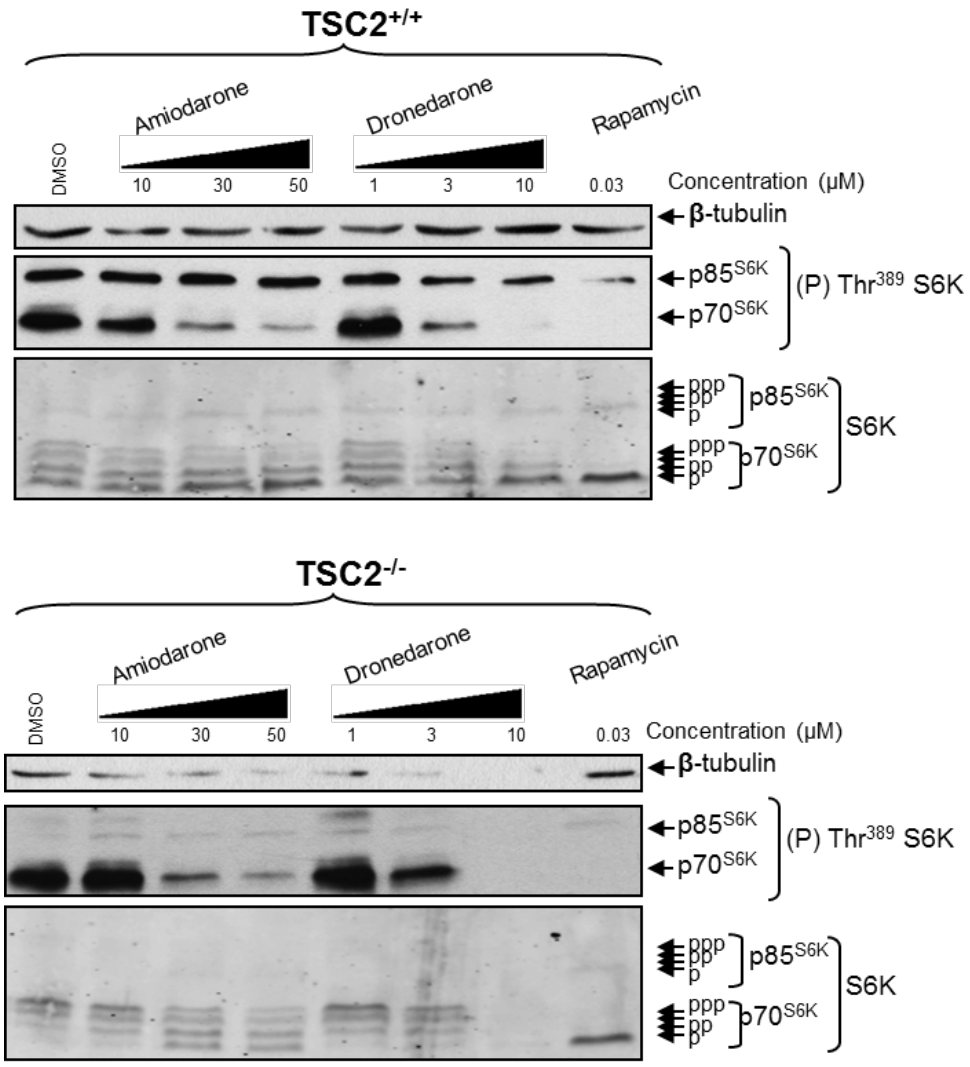


Figure 1. Effect of amiodarone and dronedarone on mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The cells were incubated for a short period of time (4 h) with the indicated concentrations of drugs and the phosphorylation of the mTORC1 substrate S6K was measured by western blotting.This experiment was carried out three times, with similar results.

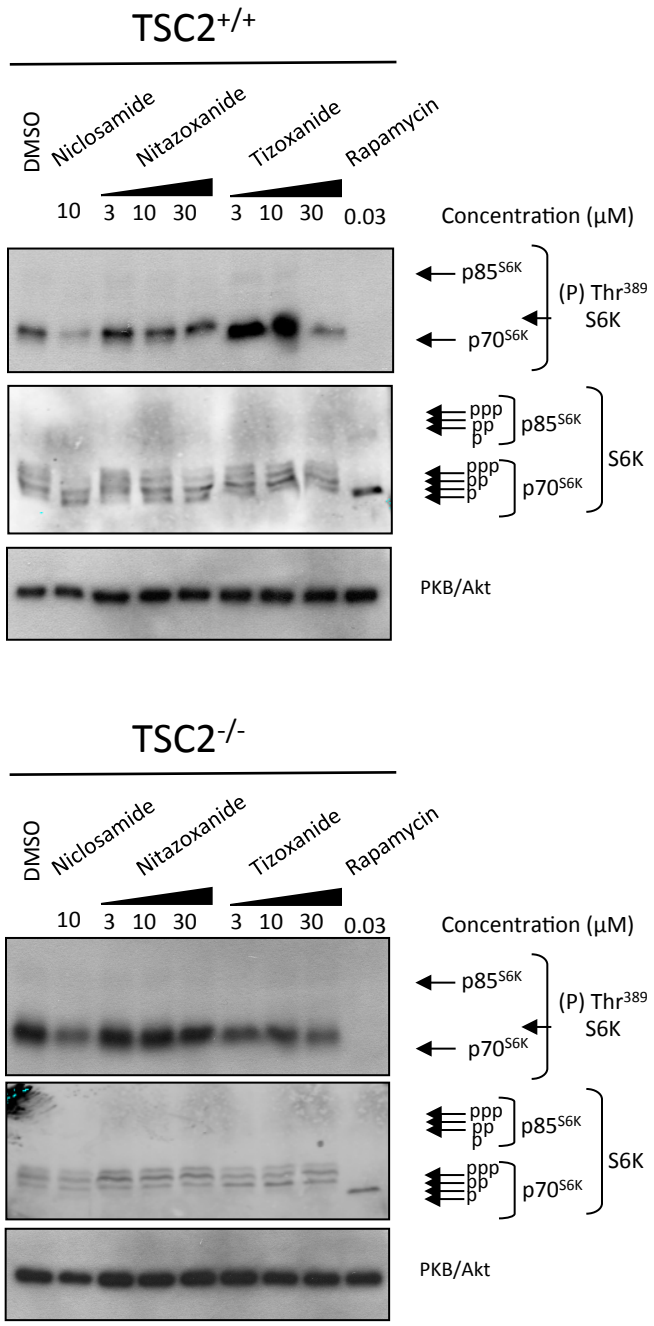


Figure 2. Effect of nitazoxanide and tizoxanide on mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The cells were incubated for a short period of time (4 h) with the indicated concentrations of drugs and the phosphorylation of the mTORC1 substrate S6K was measured by western blotting. This experiment was carried out twice, with similar results.

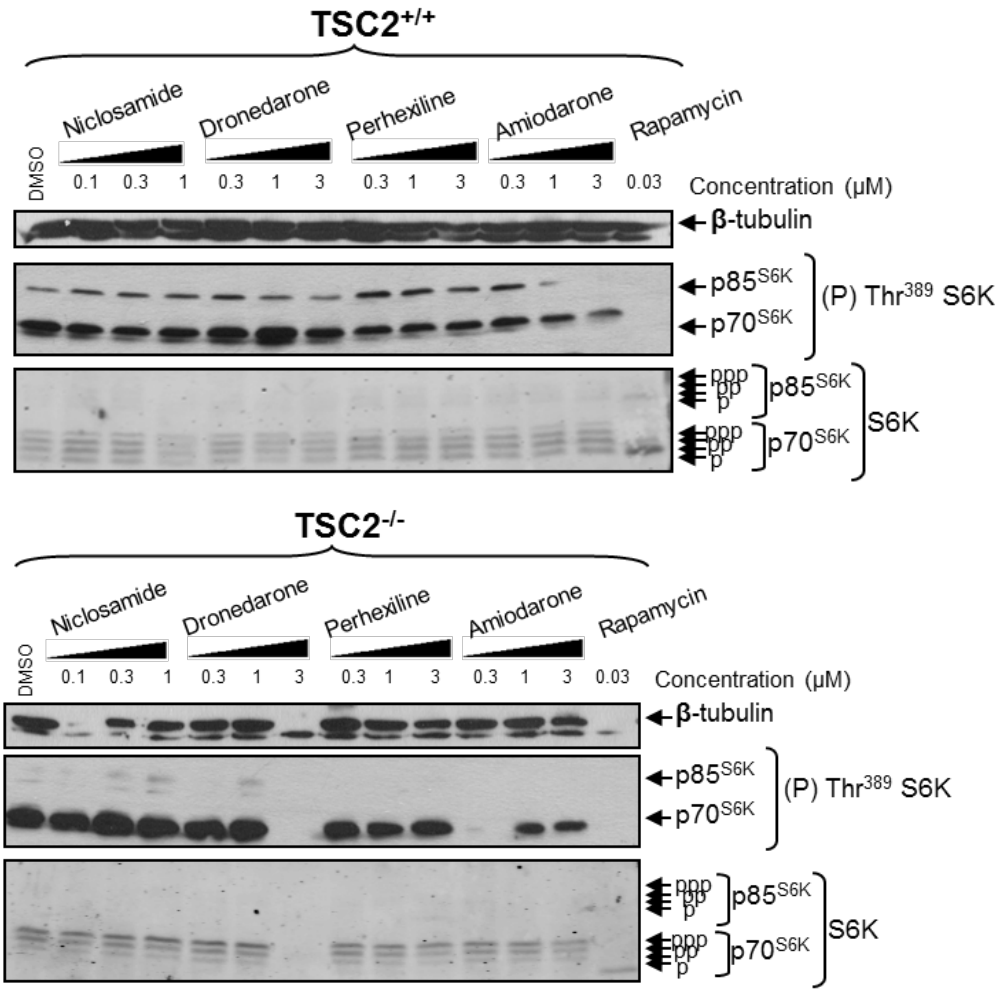


Figure 3. Effect of drugs on mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The cells were incubated for a long period of time (72 h) with the indicated concentrations of niclosamide, dronedarone, perhexiline, amiodarone or rapamycin, and the phosphorylation of the mTORC1 substrate S6K was measured by western blotting. This experiment was carried out twice, with similar results.

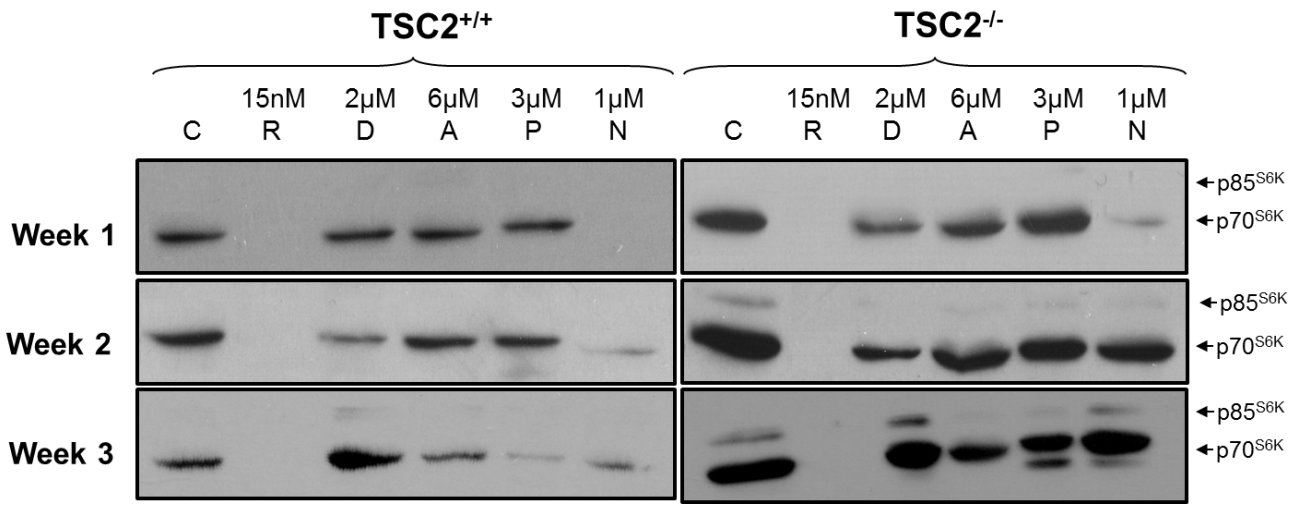


Figure 4. Effect of drugs on mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The cells were incubated for up to 3 weeks with the indicated concentrations of rapamycin, dronedarone, amiodarone, perhexiline, or niclosamide and the phosphorylation of the mTORC1 substrate S6K was measured by western blotting. This experiment was carried out twice, with similar results.

Figure 5. Effect of drugs on cell viability. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were incubated for 4 hours with the indicated micromolar concentrations of perhexiline (Per), niclosamide (Nic), amiodarore (Ami), dronedarone (Drone), rapamycin (Rap), and viable cell numbers were determined. Error bars represent positive and negative values of one standard deviation (N=3).

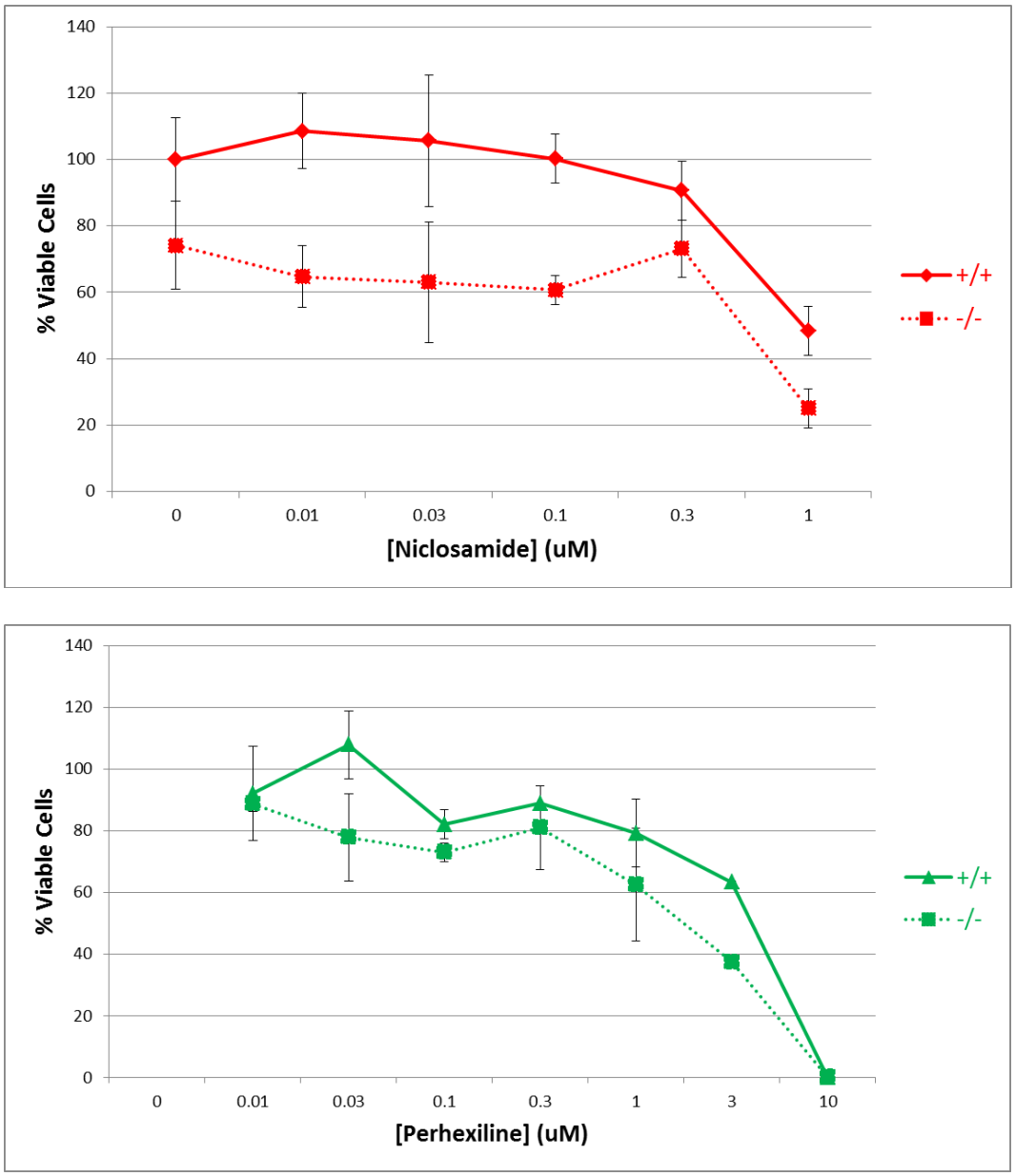
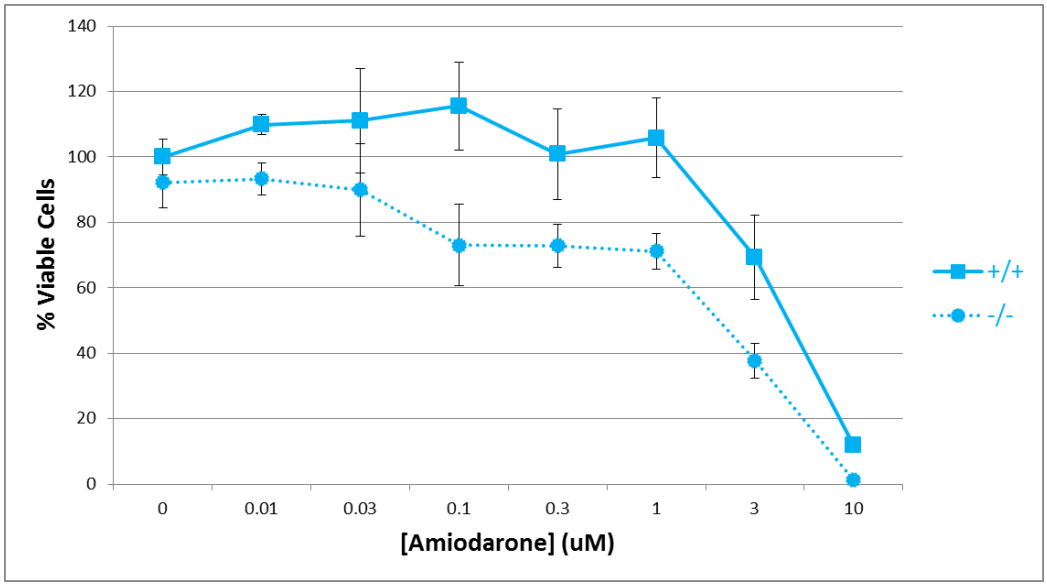


Figure 6. Effect of drugs on cell viability. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were incubated with the indicated micromolar concentrations of niclosamide or perhexiline and cell viability was measured after 5 days using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).



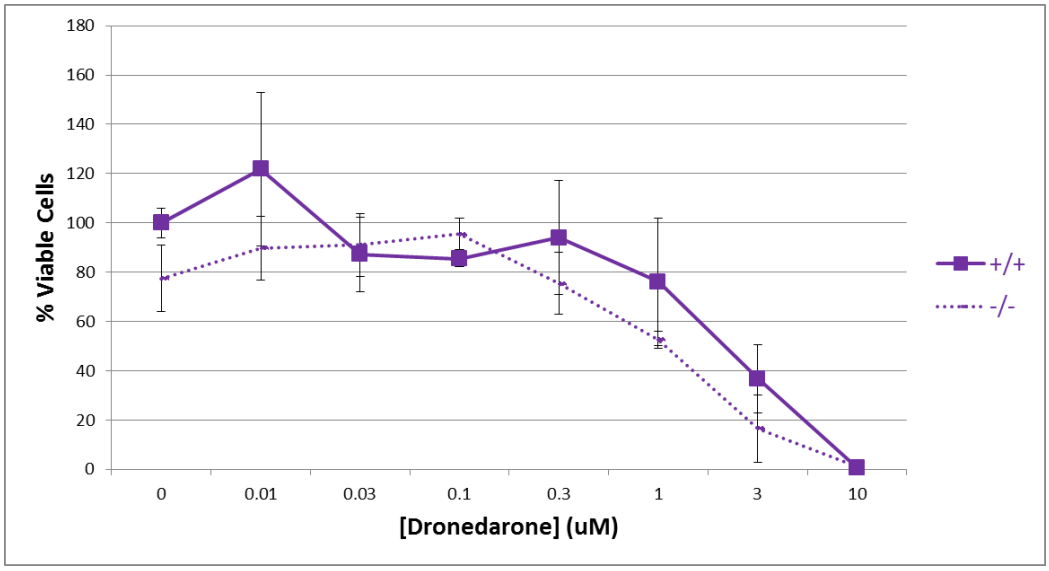


Figure 7. Effect of drugs on cell viability. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were incubated with the indicated micromolar concentrations of amiodarone or dronedarone and cell viability was measured after 5 days using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).

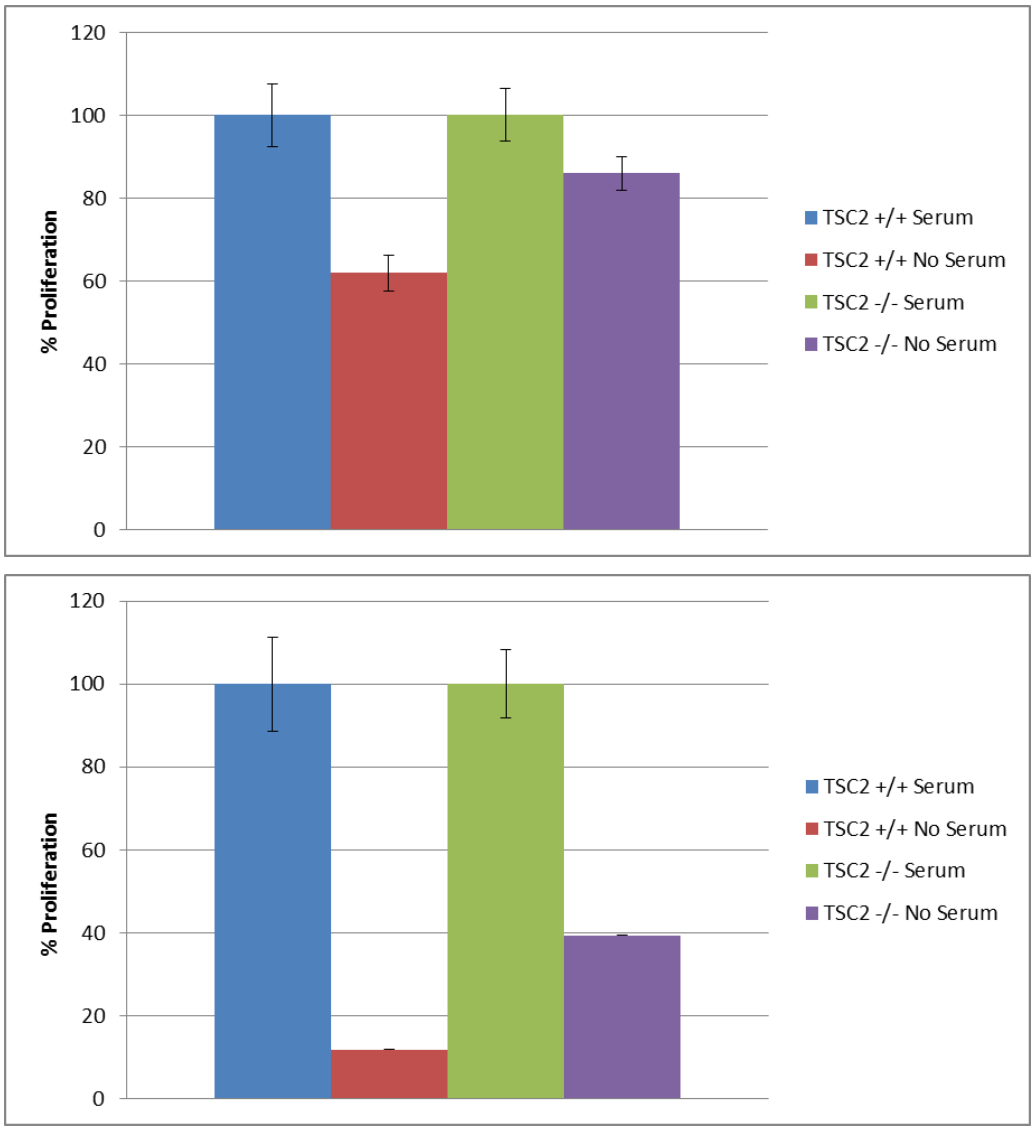


Figure 8. Effect of serum deprivation on the proliferation of $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. Cells were exposed to culture medium containing 10% serum (Serum) or 0% serum (No Serum) for 24 h (upper panel) or 48 h (lower panel) and cell viability was determined using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).

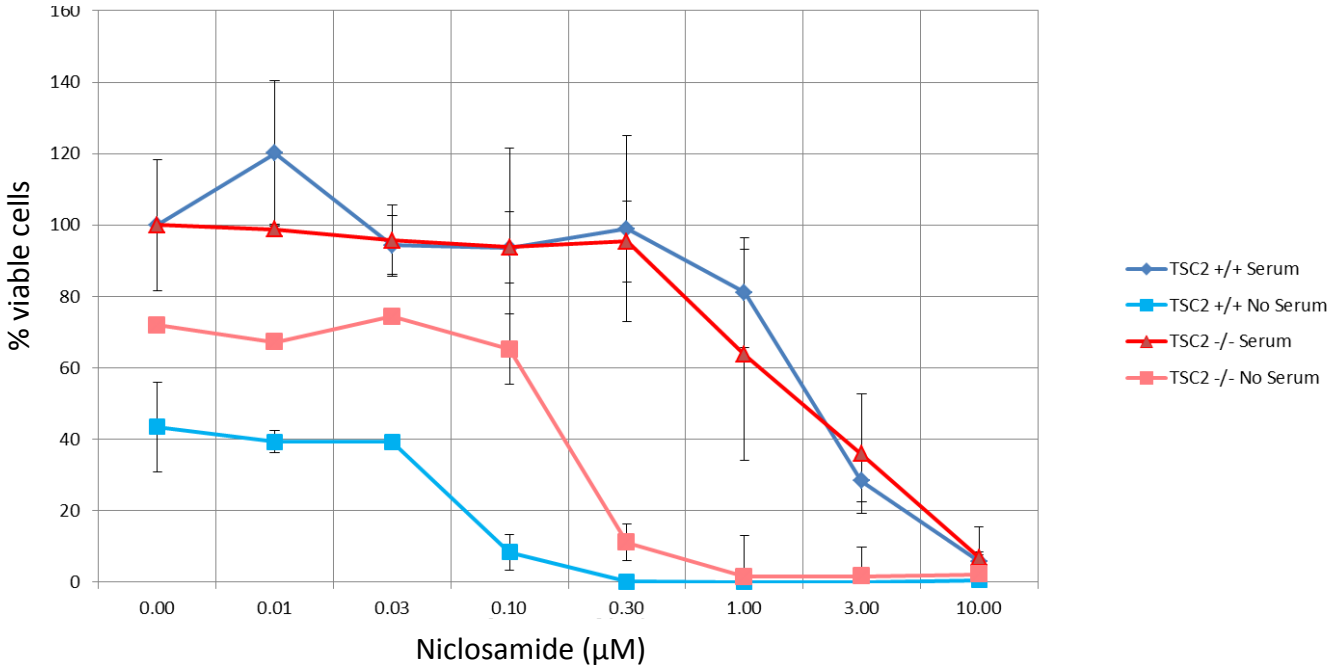


Figure 9. Effect of niclosamide on cell proliferation in the presence or absence of serum. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were exposed to the indicated concentrations of niclosamide for 48 h in medium containing 10% fetal bovine serum (Serum) or 0% serum (No Serum). Cell viability was measured using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).

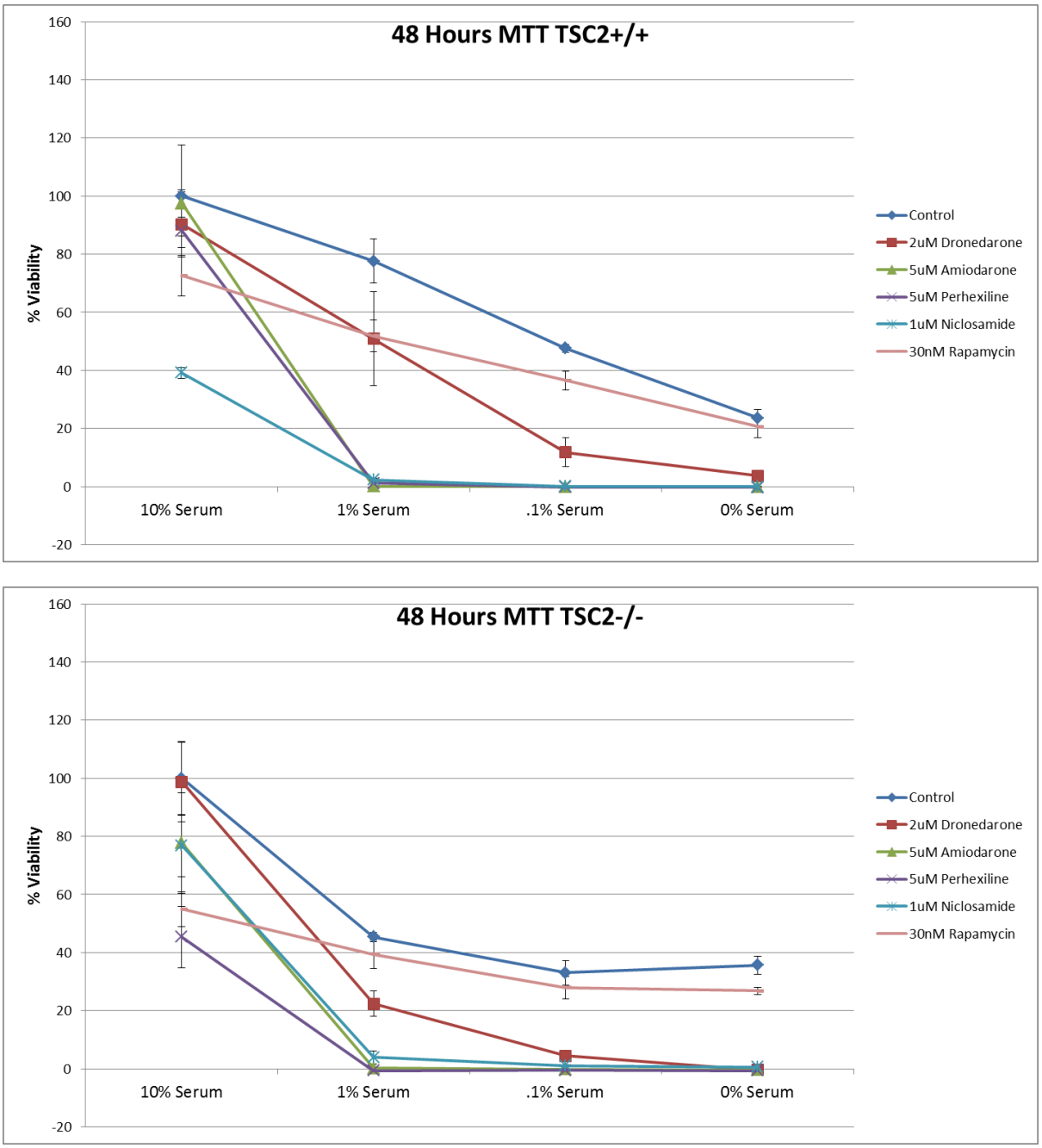


Figure 10. Effect of additional drugs on cell proliferation in the presence or absence of serum. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were exposed to the indicated concentrations of drugs for 48 h in medium containing different concentrations of fetal bovine serum (Serum). Cell viability was measured using the MTT assay. Error bars represent positive and negative values of one standard deviation (N=3).

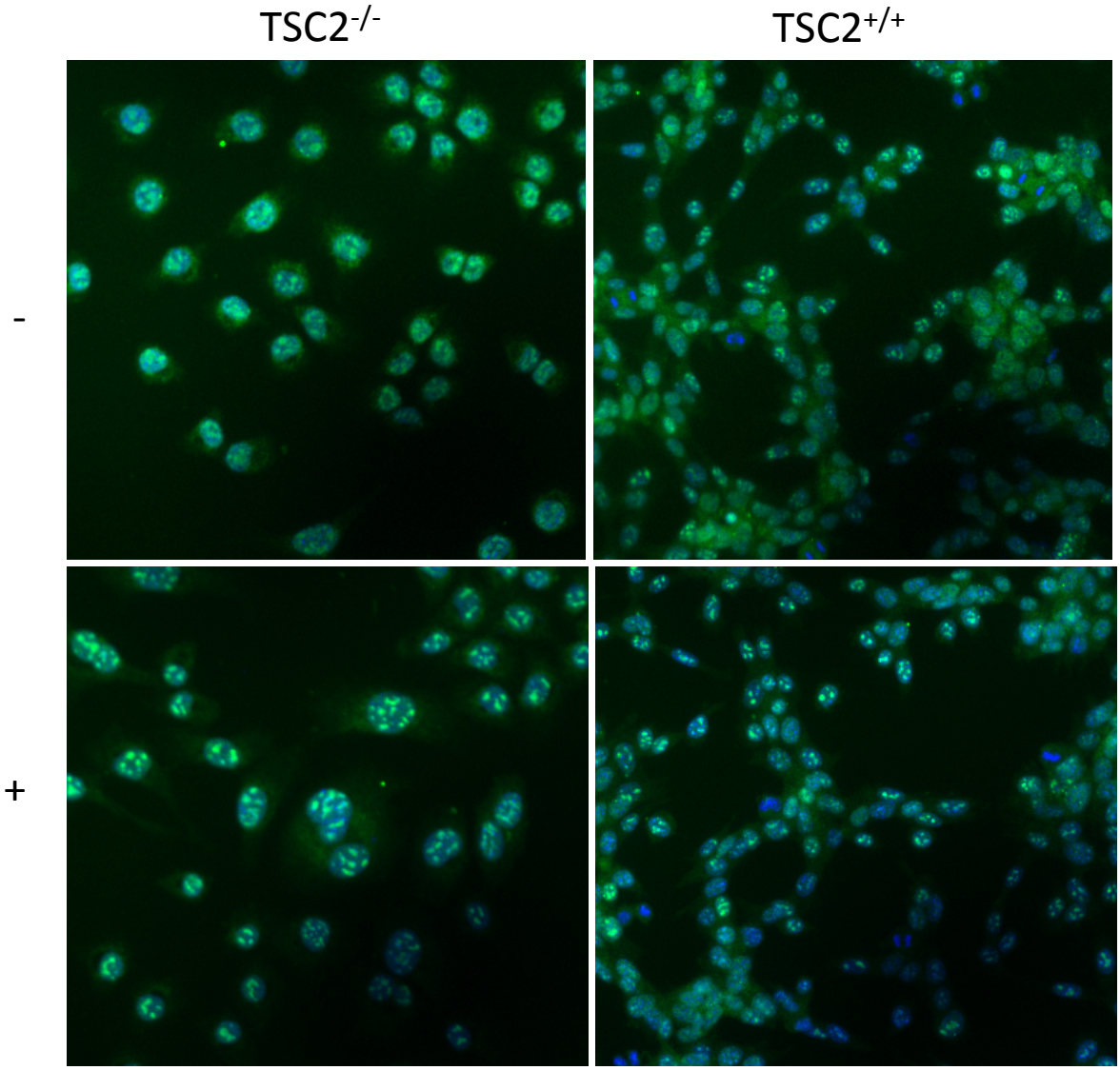


Figure 11. Effect of dronedarone on the intracellular localization of p27. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were exposed to 0 μM dronedarone (-) or 2 μM dronedarone (+) dronedarone for 4 h and p27 was detected by immunofluorescence microscopy (green). The cells were also stained with the DNA dye Hoechst 33342 to visualize nuclei (blue).

Table 1 Effect of drugs on nuclear p27 localization

	nuclear p27					Dronedarone						
	No drug	nic, 3μM	nic, 10μM	rapa, 30nM	rapa, 100nM	0.5 μM	1μM	2 μM	4μM	6 μM	8 μM	10μM
TSC2 +/+	259	605	815	244	215	267	287	351	349	441	801	289
	272	646	761	211	224	272	284	310	283	373	801	880
avg	**265**	**626**	**788**	**228**	**220**	**269**	**286**	**330**	**316**	**407**	**801**	**585**
	number of nuclei					Dronedarone						
	No drug	nic, 3μM	nic, 10μM	rapa, 30nM	rapa, 100nM	0.5 μM	1μM	2 μM	4μM	6 μM	8 μM	10μM
TSC2 +/+	2874	1585	1281	3793	3782	3447	3354	3101	3372	2406	1069	34
	3223	1817	1479	4209	3551	4014	3466	3442	3654	2953	1682	276
avg	**3049**	**1701**	**1380**	**4001**	**3667**	**3731**	**3410**	**3272**	**3513**	**2680**	**1376**	**155**

	nuclear p27					Dronedarone						
	No drug	nic, 3μM	nic, 10μM	rapa, 30nM	rapa, 100nM	0.5 μM	1μM	2 μM	4μM	6 μM	8 μM	10μM
TSC2 -/-	536	685	824	603	542	465	543	539	633	968	610	82
	661	860	948	612	591	594	547	635	690	762	829	31
avg	**598**	**772**	**886**	**607**	**566**	**529**	**545**	**587**	**662**	**865**	**720**	**56**
	number of nuclei					Dronedarone						
	No drug	nic, 3μM	nic, 10uM	rapa, 30nM	rapa, 100nM	0.5 μM	1μM	2 μM	4μM	6 μM	8 μM	10μM
TSC2 -/-	398	252	191	395	370	676	648	492	422	123	147	346
	399	222	192	437	417	498	589	646	349	328	270	123
avg	**399**	**237**	**192**	**416**	**394**	**587**	**619**	**569**	**386**	**226**	**209**	**235**

Nuclear p27 numbers are arbitrary fluorescence intensity numbers in the nuclear region defined by DNA staining.

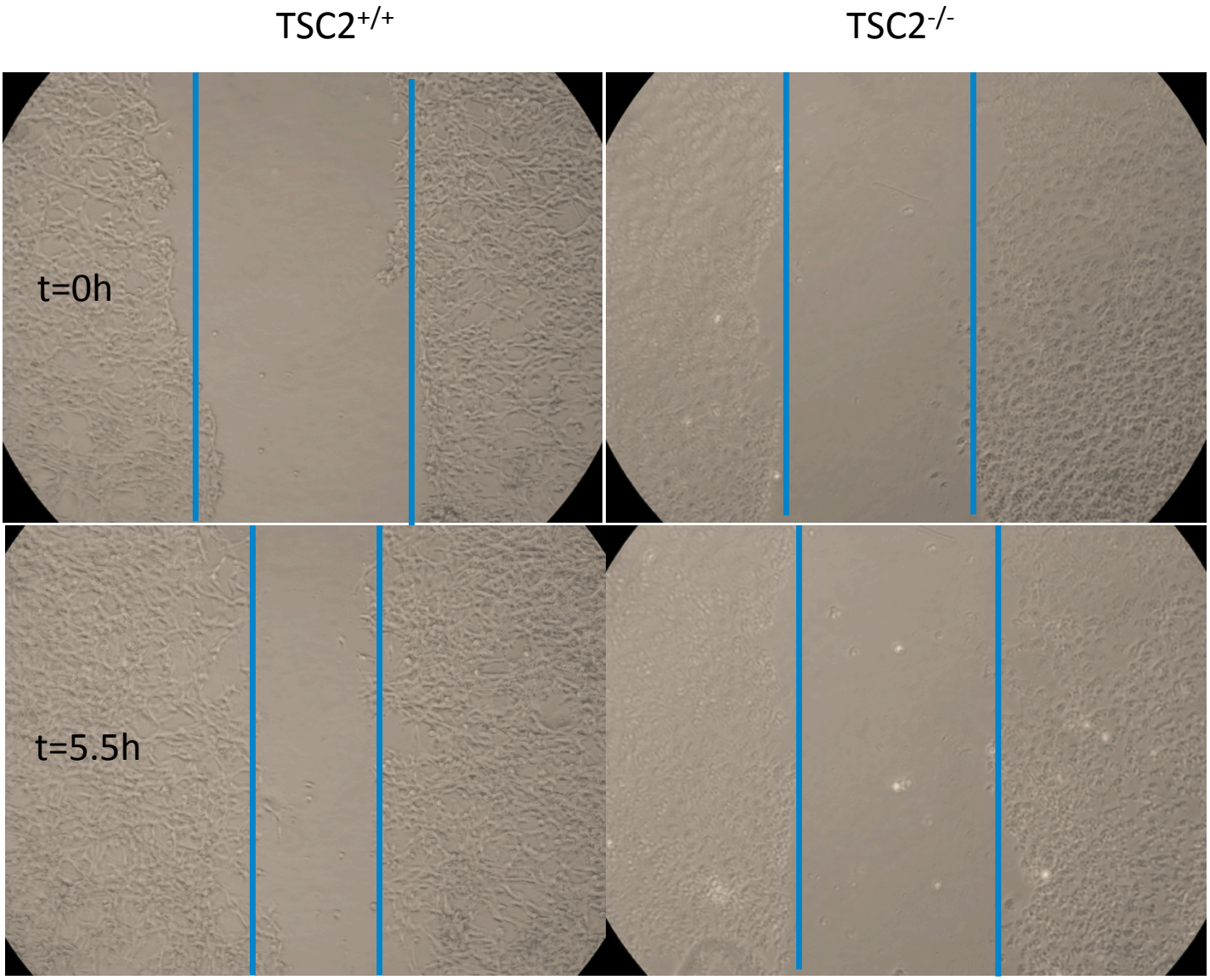
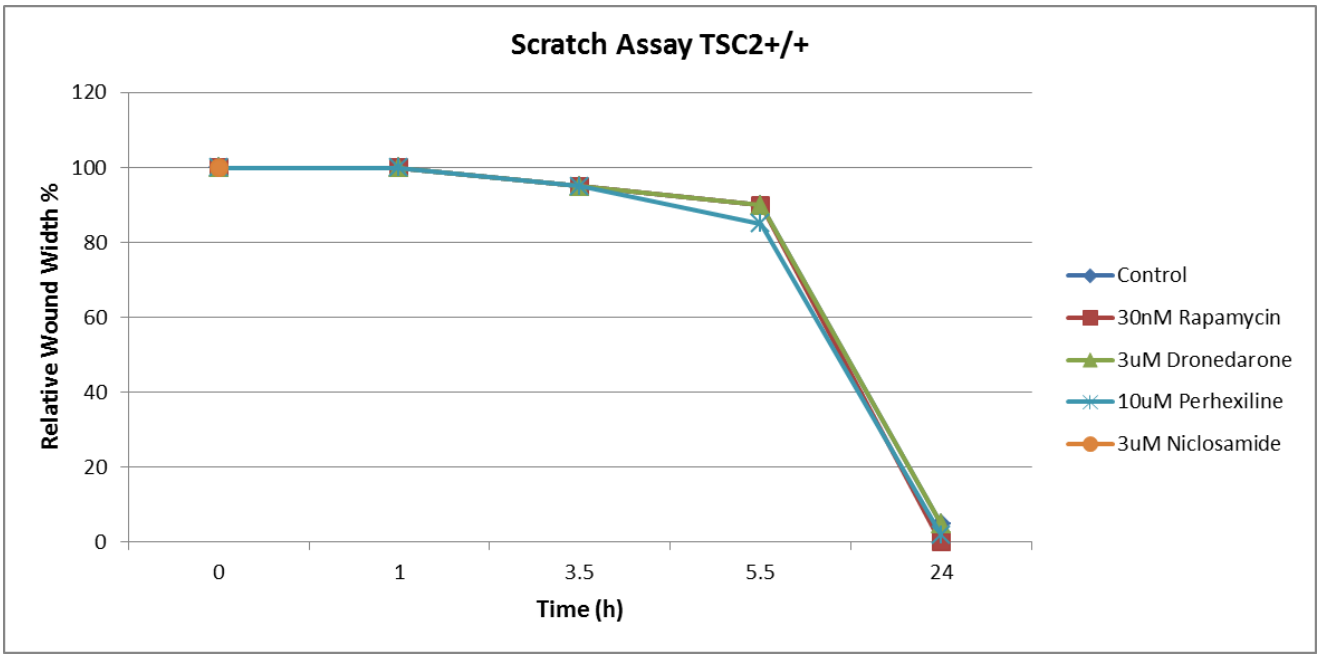


Figure 12. Wound healing assay used to examine the effect of drugs on cell motility. Confluent $TSC2^{+/+}$ and $TSC2^{-/-}$ cells monolayers were scratched to generate a gap (t=0h) and cell movement to fill the gap was monitored at t=5h in the same area. The blue bars indicate the gap boundaries.



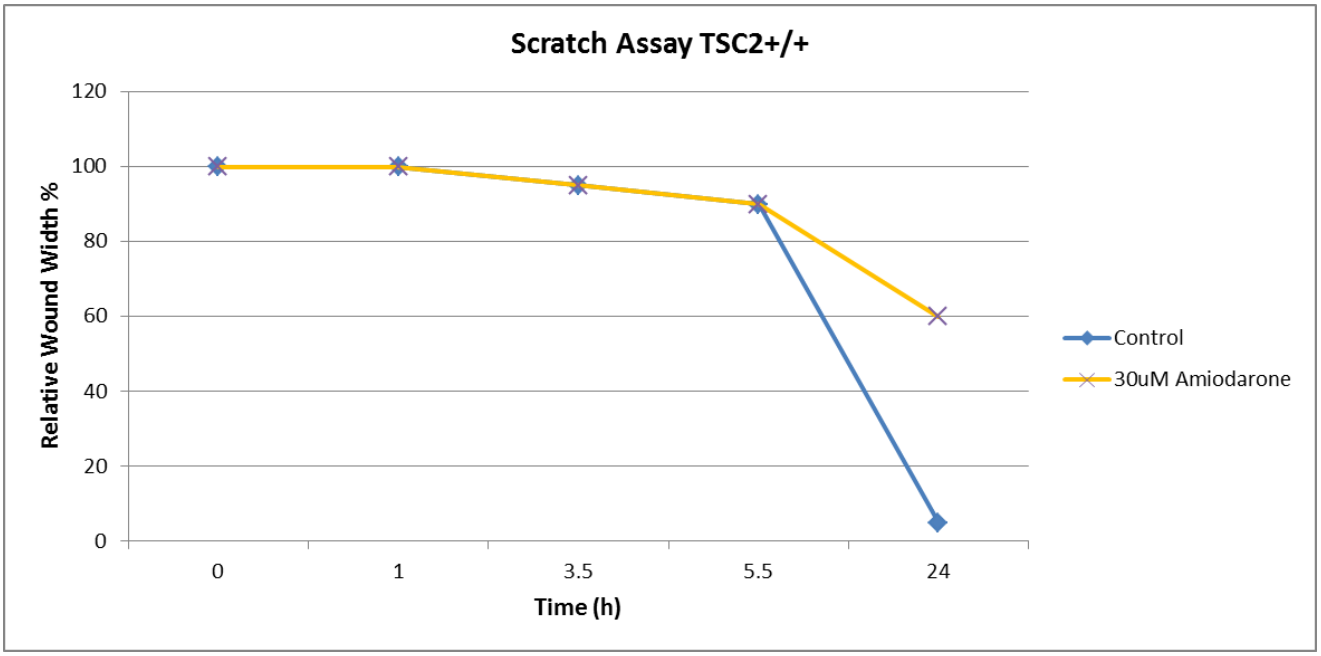
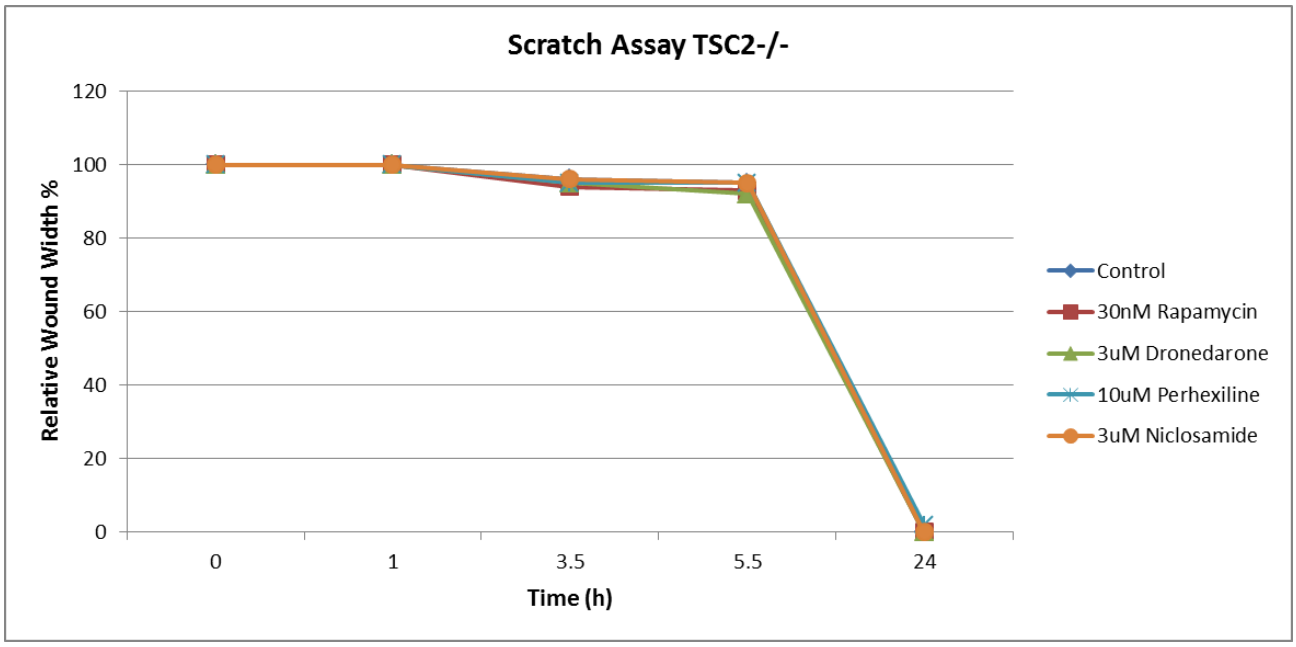


Figure 13. Effect of drugs on wound healing in $TSC2^{+/+}$ cells. Cell migration into the cleared area was measured over time after addition of drugs t= h.



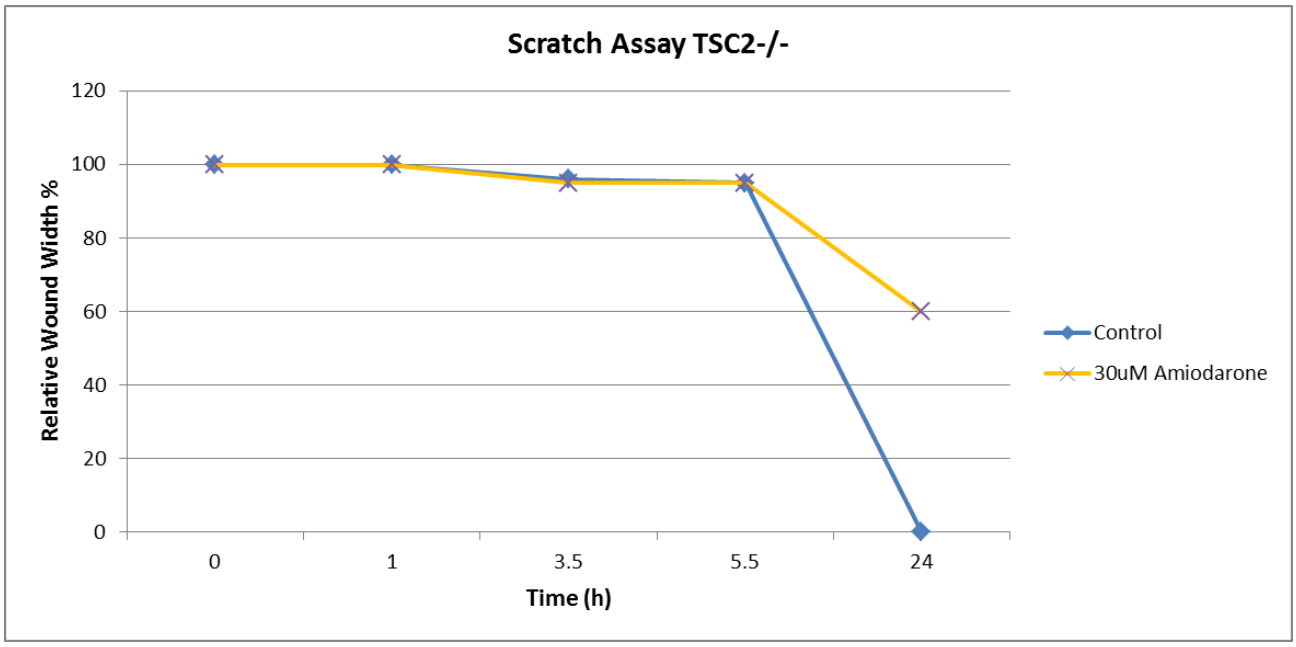


Figure 14. Effect of drugs on wound healing in TSC2$^{-/-}$ cells. Cell migration into the cleared area was measured over time after addition of drugs t=0h.

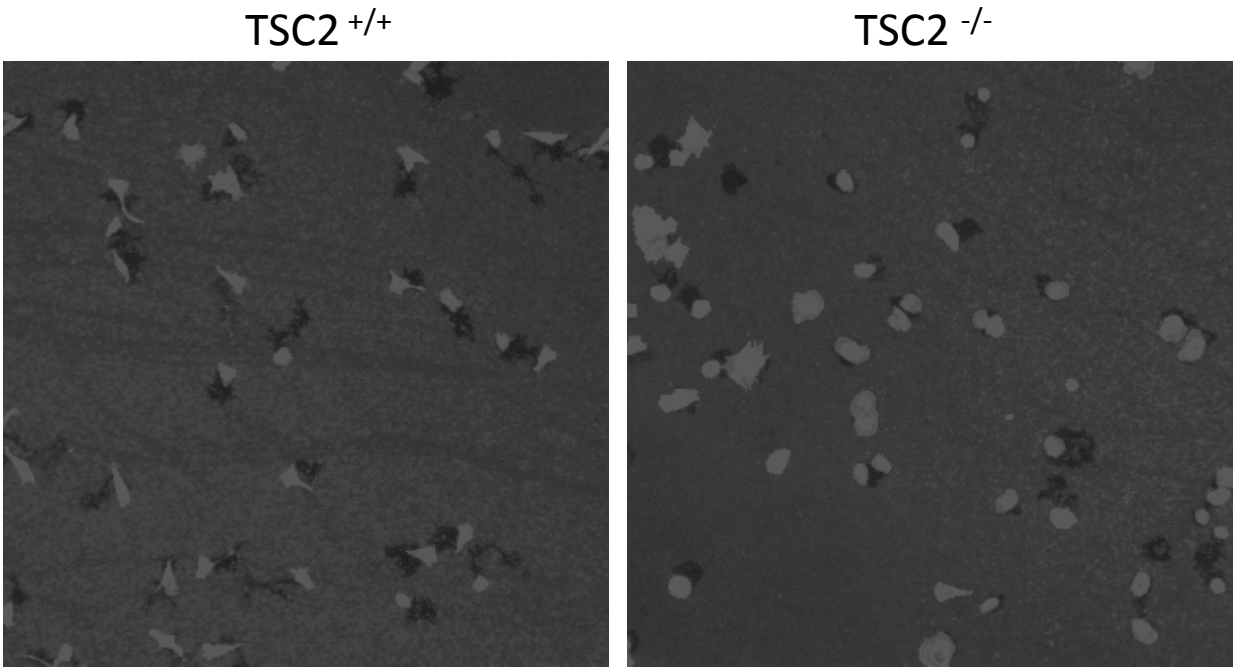


Figure 15. Fluorescent bead clearing track clearing assay used to examine the effect of drugs on cell motility. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were deposited onto a lawn of fluorescent beads. As cells moved, they phagocytosed the beads, leaving a fluorescence-free track. After 5 hours, the cells were also stained with a fluorescent dye. The area cleared of fluorescent beads was measured by automated fluorescence microscopy.

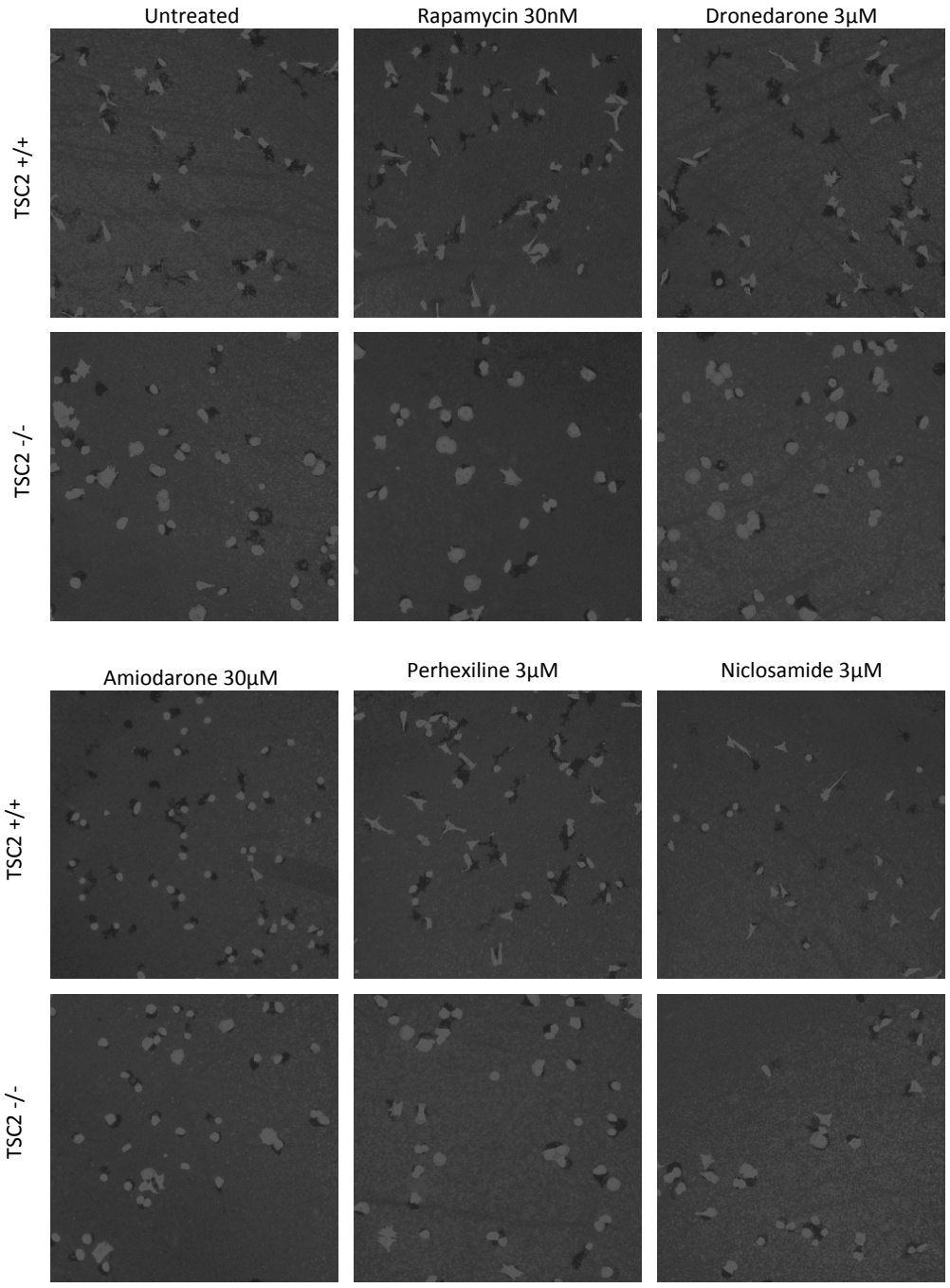


Figure 16. Effect of drugs on fluorescent bead clearing. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were deposited onto a lawn of fluorescent beads and drugs were added for 5 hours. The area cleared of fluorescent beads was measured by automated fluorescence microscopy.

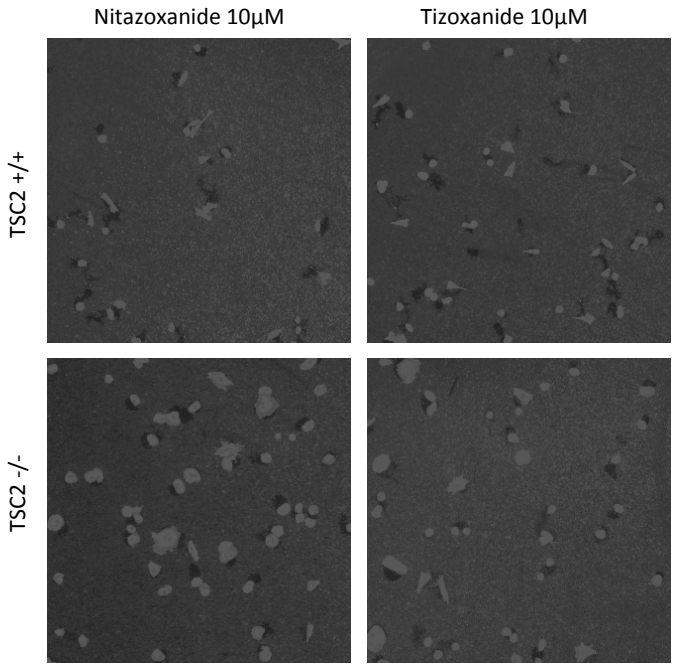


Figure 16 (continued).

Mean Motion Trac kArea

	TSC2 +/+			TSC2 -/-		
	Untreated	Rapamycin 3 nM	Rapamycin 30 nM	Untreated	Rapamycin 3 nM	Rapamycin 30 nM
	5085	3307.29	3435.38	2958.03	2253.9	2296.35
	2770.1	4104.81	3152.21	4323.79	2108.7	1842.03
Average	3927.55	3706.05	3293.80	3640.91	2181.30	2069.19
StdDev	1636.88	563.93	200.23	965.74	102.67	321.25

Mean Motion Trac kArea

	Untreated	Dronedarone 3 µM	Amiodarone 30 µM	Perhexiline 3 µM	Niclosamide 3 µM	Nitazoxanide 10 µM	Tizoxanide 10 µM
TSC2 +/+	5810.04	5535.54	4426.19	4797.36	3607.18	6127.42	5410.41
	6379.52	6847.07	3472.17	4354.51	3601.54	6871.4	
Average	6094.78	6191.31	3949.18	4575.94	3604.36	6499.41	5410.41
StdDev	402.68	927.39	674.59	313.14	3.99	526.07	0.00

	Untreated	Dronedarone 3 µM	Amiodarone 30 µM	Perhexiline 3 µM	Niclosamide 3 µM	Nitazoxanide 10 µM	Tizoxanide 10 µM
TSC2 -/-	4442.67	3741.76	3245.58	4948.39	4537	6457.65	4829.96
	4453.42	3512.36	3747.14	3920.24	4474.66	5900.65	
Average	4448.05	3627.06	3496.36	4434.32	4505.83	6179.15	4829.96
StdDev	7.60	162.21	354.66	727.01	44.08	393.86	0.00

Figure 17. Effect of drugs on fluorescent bead clearing. The area cleared of fluorescent beads (Mean Motion Track Area, arbitrary units) was measured by automated microscopy from duplicate samples.

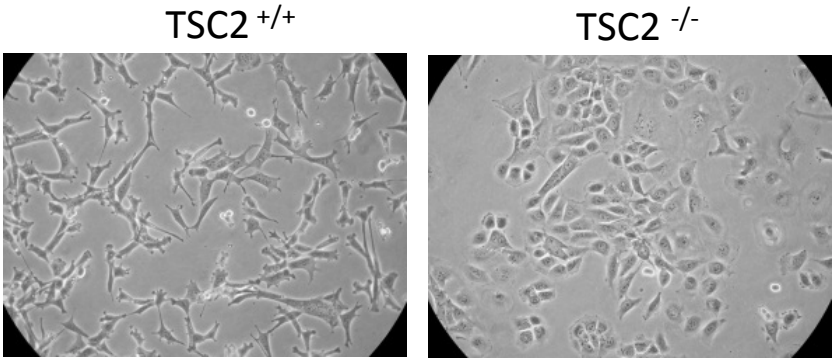


Figure 18. Morphology of $TSC2^{+/+}$ and $TSC2^{-/-}$ mouse embryo fibroblasts 20x magnification at ~50% confluency.

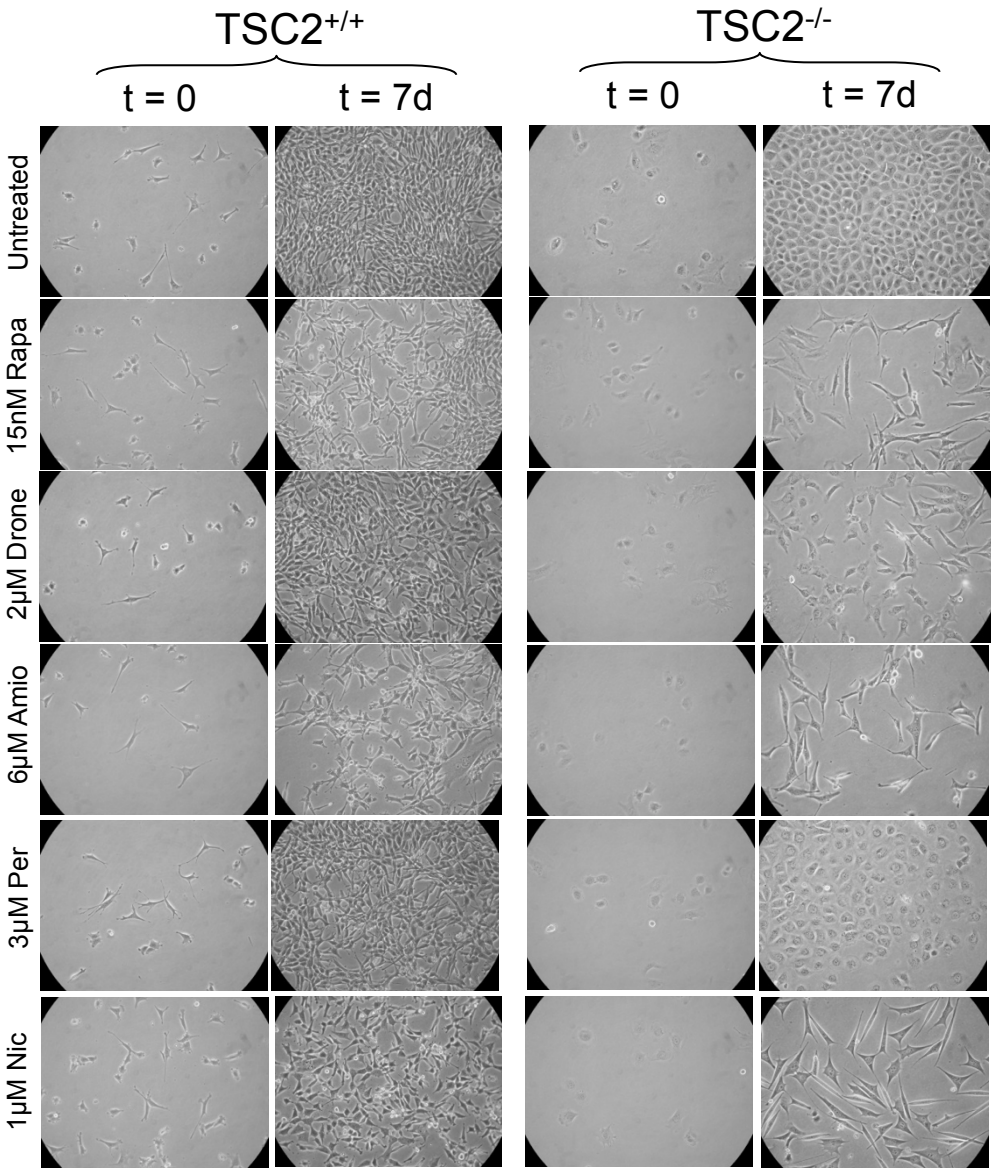


Figure 19. Effect of drugs on morphology of $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. Pictures taken at 20x magnification.

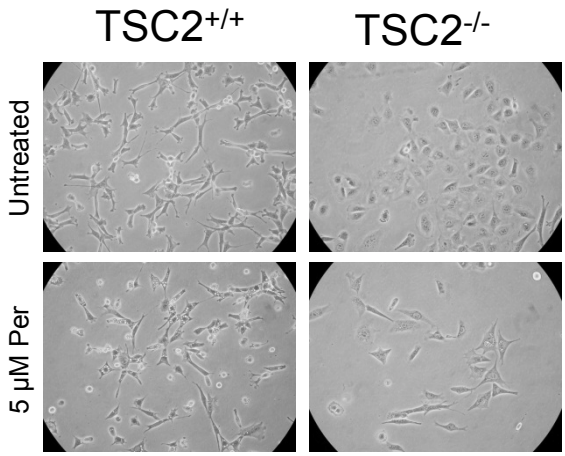


Figure 20. Effect of 5 µM perhexiline for 48 h on morphology of $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. Pictures taken at 20x magnification.

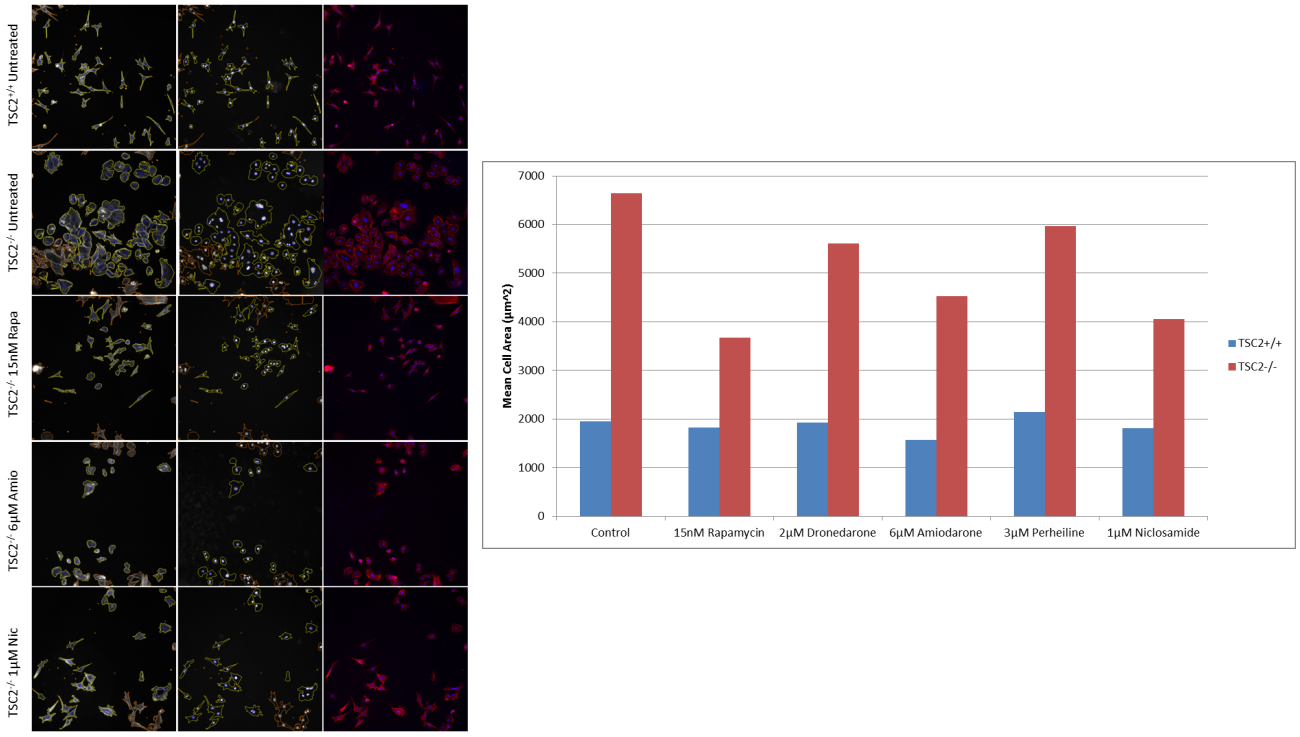


Figure 21. Quantification of mean cell size of $TSC2^{+/+}$ and $TSC2^{-/-}$ cells after 7 days drug treatment. Example pictures displayed using rhodamine phalloidin to stain F-Actin (red). Cells were also stained with the DNA dye Hoechst 33342 to visualize nuclei (blue). Mean cell area was quantified using a Cellomics Arrayscan VTI fluorescence imager.

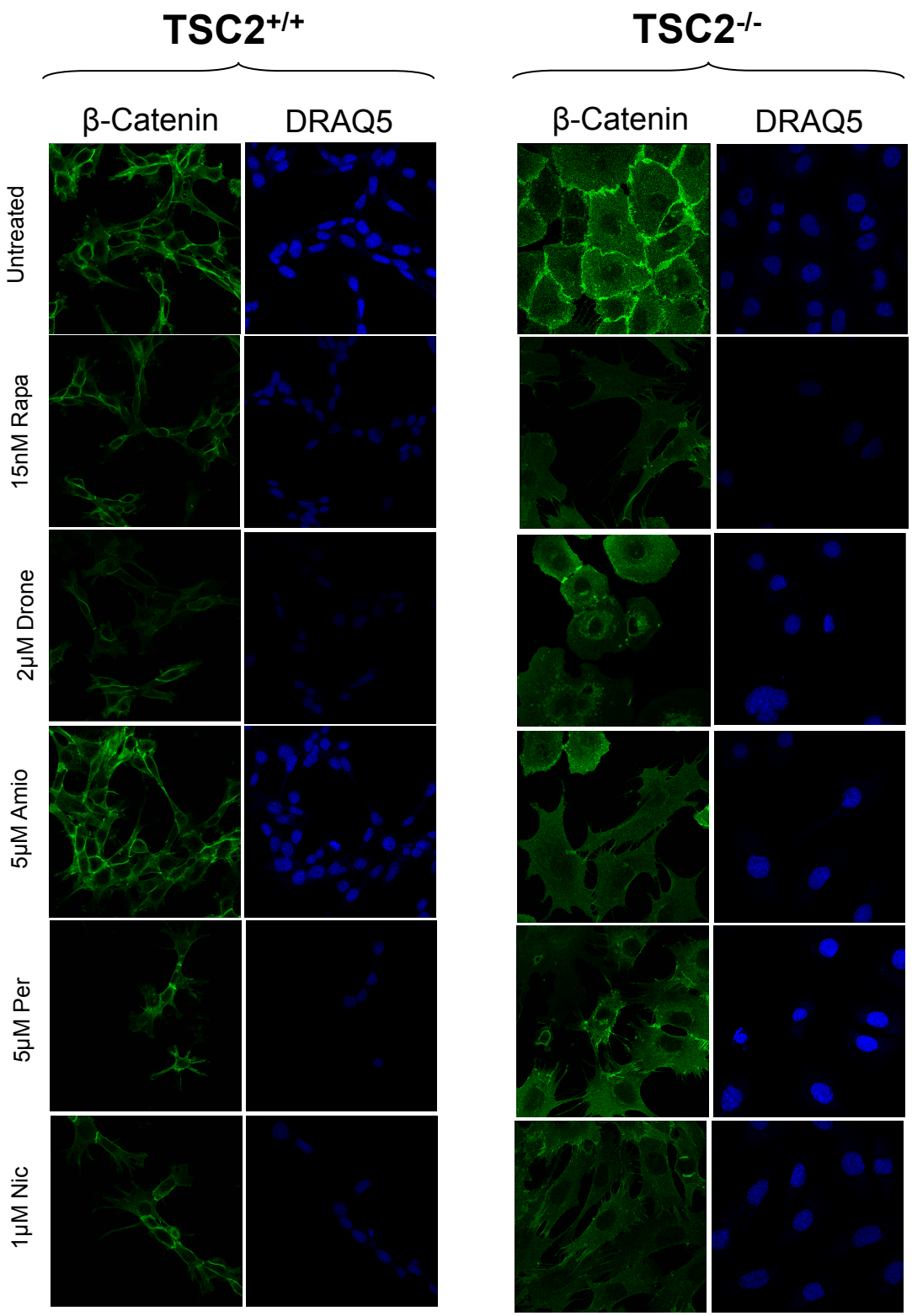


Figure 22. Effect of drugs on cell-cell contacts. $TSC2^{+/+}$ and $TSC2^{-/-}$ cells were exposed to drugs for 5 days, and β-Catenin was detected by immunofluorescence confocal microscopy (green). The cells were also stained with the DNA dye DRAQ5 to visualize nuclei (blue).

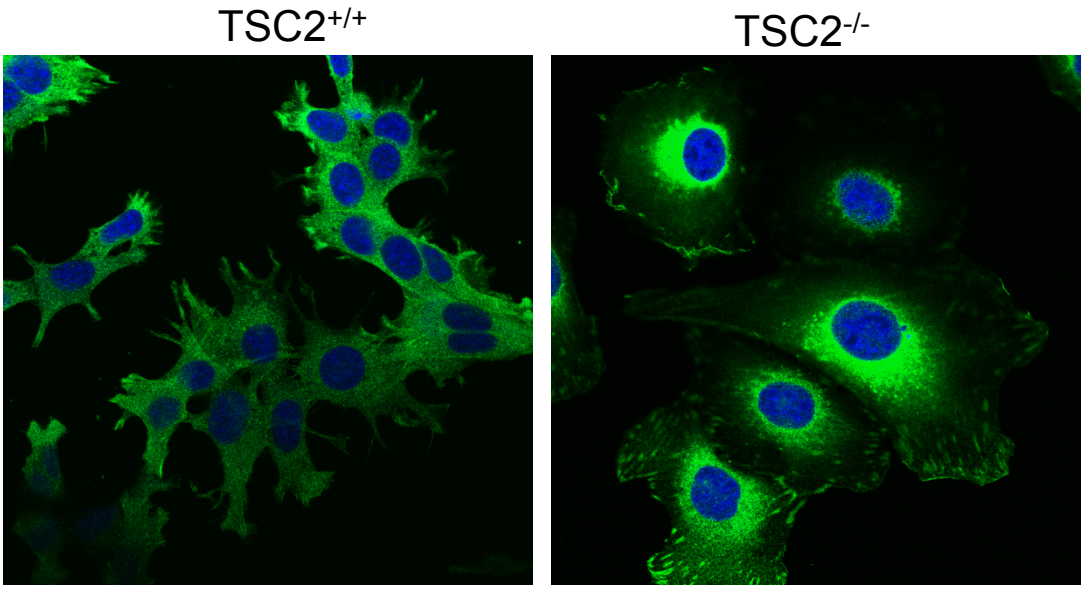


Figure 23. Visualizing focal adhesions in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. FAs were detected by immunofluorescence confocal microscopy staining for vinculin (green). The cells were also stained with the DNA dye DRAQ5 to visualize nuclei (blue).

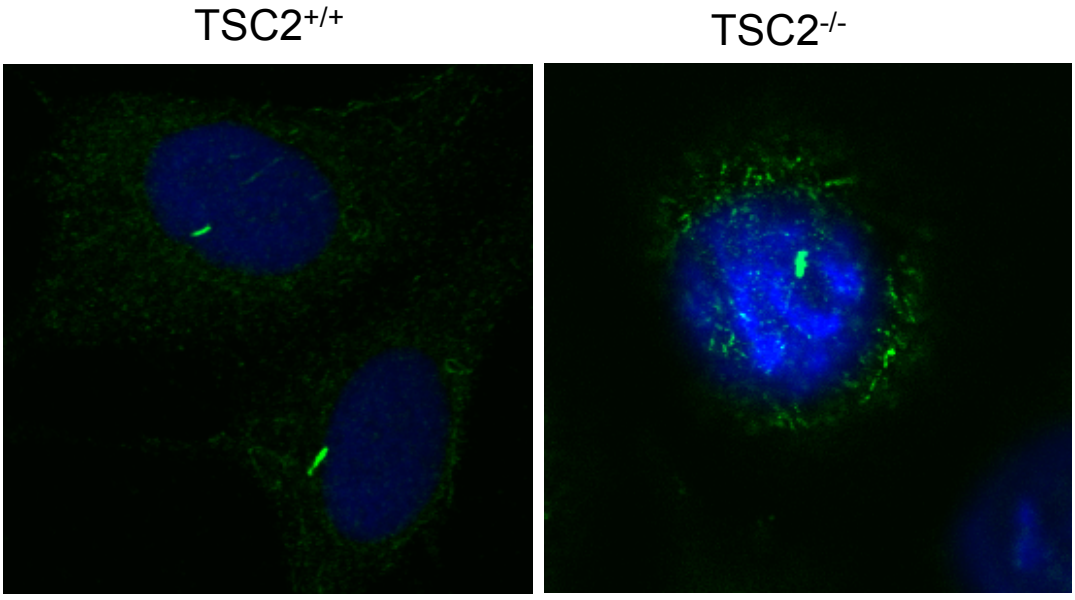


Figure 24. Visualizing the primary cilium in $TSC2^{+/+}$ and $TSC2^{-/-}$ cells. The primary cilium was detected by immunofluorescence confocal microscopy staining for acetylated tubulin (green). The cells were also stained with the DNA dye DRAQ5 to visualize nuclei (blue).

Key research accomplishments

- Identification of approved drugs capable of inhibiting mTORC1 signaling and reversing the abnormal epithelial-like phenotype of $TSC2^{-/-}$ cells.

Reportable outcomes

None

Conclusions

In summary, niclosamide, perhexiline, amiodarone and dronedarone inhibit mTORC1 signaling in $TSC2^{-/-}$ cells during short term exposure. During long term exposure to the drugs, mTORC1 signaling goes back to levels seen in untreated cells, indicating that the cells activate compensatory mechanisms. Two drugs, nitazoxanide and its active metabolite tizoxanide did not significantly inhibit mTORC1 signaling. Interestingly, niclosamide, perhexiline, amiodarone and dronedarone clearly ameliorated an abnormal phenotype of $TSC2^{-/-}$ cells. They reverted the epithelial morphology of $TSC2^{-/-}$ cells to a more normal looking fibroblastic morphology and reduced the abnormally high cell-cell contacts seen in $TSC2^{-/-}$ cells. It is not known whether reverting this phenotype would be sufficient to ameliorate the condition of tuberous sclerosis patients.

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Appendices

none